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LETTER

SYBR Green I-based product-enhanced reverse transcriptase assay for quantification of retroviral PFV and detection of the divalent cation preference of PFV RT

Dear Editor,

Prototype foamy virus (PFV) belongs to the genus *Spumavirus* in the *Spumaretrovirinae* subfamily of *Retroviridae*. Although PFV and HIV have much in common, research into PFV has lagged far behind that into HIV, as PFV appeared to be non-pathogenic both in accidentally infected humans and in experimentally infected animals. In recent decades, however, more attention has been focused on PFV because it seems to be a promising candidate vector for gene therapy in clinical applications. Compared with other retroviral vectors, vectors derived from PFV appear to be safer (Sastry L, et al., 2005). In addition, it was found that PFV vectors are efficient vehicles for stable gene transfer in proliferating cells, and that the transferred genes are able to achieve stable integration and long-term expression in the target cells.

To expedite the development and application of PFV vectors, it is essential to have a rapid, sensitive, and accurate assay to monitor the quality and quantity of PFV virions (Vermeire J, et al., 2012). The traditional method was to observe the cytopathic effect (CPE), but this is time-consuming, labor-intensive, and relatively insensitive. Furthermore, PFV may not be cytopathic in some types of cells. Quantitative real-time PCR (qPCR) is sensitive and reproducible, but the result it gives can be hundreds of times higher than the actual value (Geraerts M, et al., 2006). An assay based on enhanced green fluorescent protein (eGFP) fluorescence and fluorescent-activated cell sorting (FACS) is the most straightforward technique to quantify infective virions, but it cannot discriminate cells with single or multiple integrations. An alternative PFV titration method, called product-enhanced reverse transcriptase (PERT) assay, is based on the quantification of reverse transcriptase (RT) activity. The newest PERT assays are based on real-time PCR, which can detect 1–10 retrovirus particles (Fan X Y, et al., 2006), whereas conventional PERT assays are only able to detect 10^4–10^6 retrovirus particles in a short linear range (Khan A S, et al., 1999; Sears J F, et al., 1999; Andre M, et al., 2000).

In this study, a SYBR Green-based PERT (SG-PERT) reaction was established to determine PFV titer. Purification of PFV virions was performed as described previously (Sun Y, et al., 2007). A sufficient amount of exogenous RNA from bacteriophage MS2 (GenBank accession No.V00642) was used as template, as MS2 is a RNA virus without a DNA phase in its life cycle, which excluded the possibility of using homologous DNA. AMV RT (Promega, Madison, WI, USA) was used to establish a standard curve so that the level of PFV virions could be detected accurately and sensitively.

Because AMV RT and PFV RT are two different types of RT enzymes derived from different kinds of viruses, and thus might possess diverse optimum temperatures in reverse transcription reactions, the reaction temperatures were first optimized. The AMV RT dilutions and PFV lysates were prepared as described previously (Ma Y K, et al, 2009). The reverse transcription reaction mixture was composed of 30 ng/μL bacteriophage MS2 genomic RNA template, 1 μmol/L primer qMS276-297 (5′-GTAGTGC-CACTGTTTCGTTTTG-3′), 250 μmol/L dNTPs, 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 8 mmol/L $MgCl_2$, 10 mmol/L DTT, and either 10^{-3} U AMV RT or 1 μL of the virus lysate (containing the PFV RT) in a 10 μL final volume. The reactions were incubated using a temperature gradient from 37 °C to 58 °C for 1 h, followed by 95 °C for 5 min to inactivate the RT (AMV or PFV). The real-time PCR reaction mixture included 1×TransStart Top Green Mix, 0.2 μmol/L primer qMS21-42 (5′-TCCTGCTCAACTTCCTGTCGAG-3′) and 0.15 μmol/L qMS276-297, then 1 μL of the synthesized cDNA was added into the mixture to give a total volume of 20 μL. The cDNA template was omitted from the negative control. The reactions were performed with 45 cycles of amplification, comprising 5 s of denaturation at 95 °C, 20 s of annealing at 65 °C, 20 s of extension at 72 °C, and 9 s of acquisition at 80 °C. Results showed that AMV RT possessed its highest activity at 37 °C, while for PFV RT, the optimum temperature was 50 ºC (Figure 1A). Thus, 37 °C was chosen for setting up the SG-PERT standard curve, and 50 ºC was employed to quantify PFV virions. Melting curve analysis of the real-time PCR products

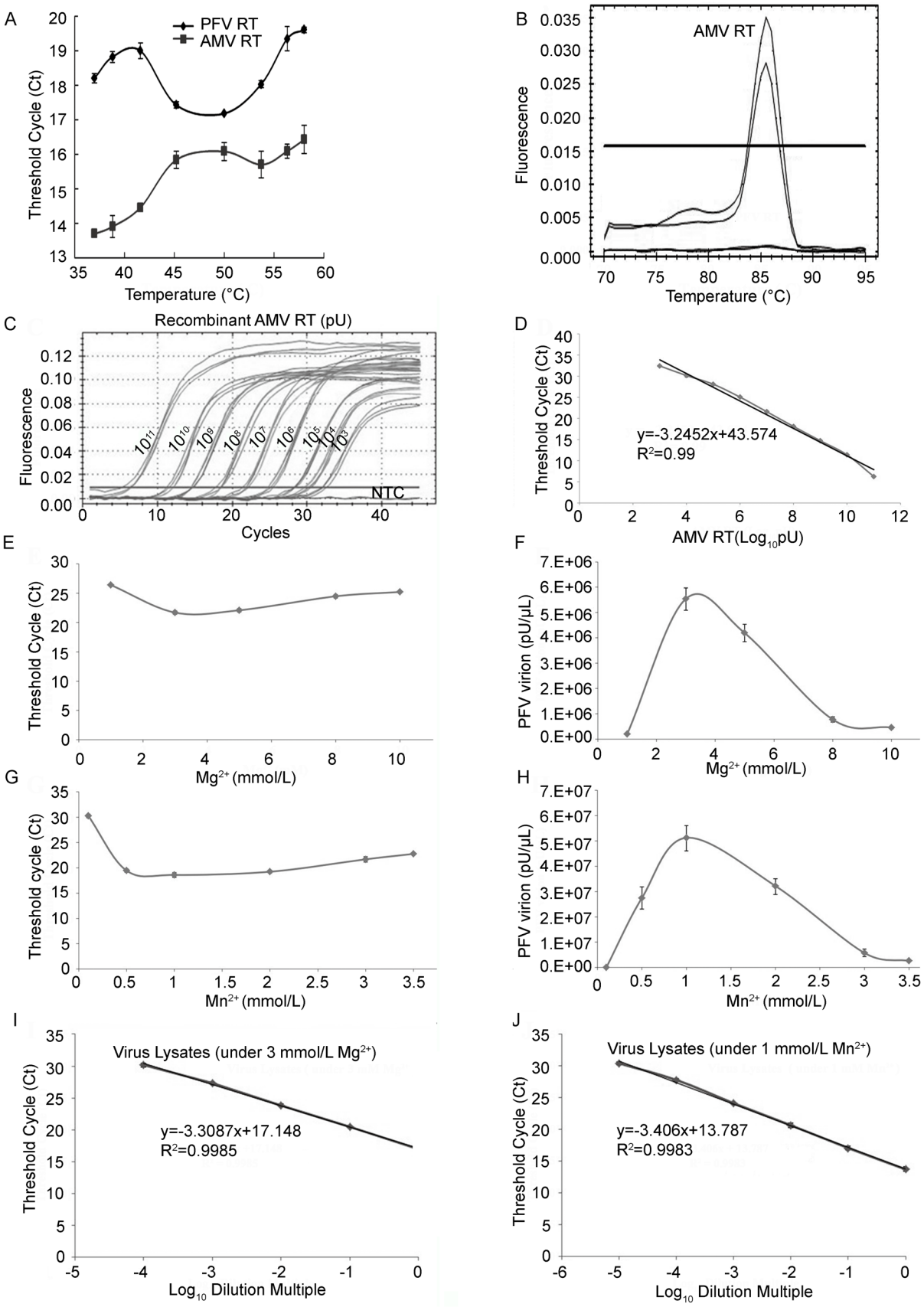
A
PFV RT
AMV RT
Threshold Cycle (Ct)
Temperature (°C)
B
AMV RT
Fluorescence
Temperature (°C)
C
Recombinant AMV RT (pU)
Fluorescence
Cycles
NTC
D
Threshold Cycle (Ct)
y=-3.2452x+43.574
R²=0.99
AMV RT(Log10pU)
E
Threshold Cycle (Ct)
Mg2+ (mmol/L)
F
PFV virion (pU/μL)
Mg2+ (mmol/L)
G
Threshold cycle (Ct)
Mn2+ (mmol/L)
H
PFV virion (pU/μL)
Mn2+ (mmol/L)
I
Virus Lysates (under 3 mmol/L Mg2+)
y=-3.3087x+17.148
R²=0.9985
Threshold Cycle (Ct)
Log10 Dilution Multiple
J
Virus Lysates (under 1 mmol/L Mn2+)
y=-3.406x+13.787
R²=0.9983
Threshold Cycle (Ct)
Log10 Dilution Multiple

Figure 1. Determination of the temperature and divalent cation preference for reverse transcriptases, establishment of the SG-PERT standard curve, and quantification of PFV virion by SG-PERT assays. A: Threshold cycles of the real-time PCR reactions using the cDNA templates reverse-transcribed by AMV RT or PFV RT in the range 37–58 °C. The optimum temperatures for AMV RT and PFV RT were 37 °C and 50 °C, respectively. B: Melting curves of the real-time PCR products. The distinct melting peak at 85.5 °C for both RTs verified the specificity of the real-time PCR reaction. C: Amplification curves obtained with the cDNA templates reverse-transcribed by 10-fold serial dilutions of AMV RT. D: The linear standard curve of the SG-PERT assay established based on the data from (C). The correlation coefficient reached 0.99 in a wide linear range within 7 logs (from 4 to 10), which indicated a favorable linearity of the SG-PERT standard curve. E–G: Threshold cycles of the real-time PCR reactions using the cDNA templates reverse-transcribed by PFV RT with Mg^{2+} (E) or Mn^{2+} (G), and the amounts of PFV RT detected under different concentrations of Mg^{2+} (F) or Mn^{2+} (H). I, J: It was clear that the optimum concentrations of Mg^{2+} or Mn^{2+} for PFV RT were 3 mmol/L and 1 mmol/L, respectively, with the maximum level of 5.54×10^6 pU/μL of PFV RT detected with 3 mmol/L Mg^{2+}, and 5.12×10^7 pU/μL detected with 1 mmol/L Mn^{2+}. Threshold cycles of the real-time PCR reactions using the cDNA templates reverse-transcribed by the 10-fold serial dilutions of the virus lysates (containing PFV RT) with Mg^{2+} (I) or Mn^{2+} (J). The linear curves had high correlation coefficients, reaching 0.99, proved that both Mg^{2+}-based and Mn^{2+}-based SG-PERT assays had a favorable amplification efficiency and linearity.

confirmed the presence of the specific DNA fragment with an identical melting peak at 85.5 °C (Figure 1B).

Levels of AMV RT enzymes ranging from 10^3 to 10^{11} pU were then used to set up the standard curve. The results showed that amplification of the MS2 substrate correlated with the input amount of AMV RT (Figure 1C). The standard curve was then generated according to the quantity of AMV RT and the corresponding Ct value (Figure 1D). The inner-assay coefficient of variation (CV) and the inter-assay CV of the standard curve were no more than 6%, which indicated high reproducibility of the experiments (data not shown). In addition, a high correlation coefficient (0.99) of the curve was obtained with a wide linear range of 7 logs, which displayed a favorable linearity. The detection sensitivity of AMV RT was determined as 10^3 pU, which was equivalent to 100 AMV RT molecules (Ma Y K, et al, 2009) or about a particle of AMV (which contains 70 RT molecules) (Panet A, et al., 1975) or HIV-1 (which contains 80 RT molecules) (Layne S P, et al., 1992). The efficiency of the reaction was 103% [$Eff=-1+10^{(-1/slope)}$], indicating high accuracy, sensitivity, and reproducibility of the SG-PERT assay with AMV RT. Thus, the standard curve could be used to convert the test article threshold cycles to RT activity.

All retroviruses can be divided into two categories based on the presence of either an Mg^{2+}-requiring RT or an Mn^{2+}-requiring RT (Khan A S, et al., 1999). Previous studies showed that PFV have a preference for Mn^{2+} (Liu W T, et al., 1977); however, the divalent cation preference of PFV RT needs confirmation, and the optimal concentrations of Mg^{2+} and Mn^{2+} for PFV RT are still unclear. Hence, we used the SG-PERT assay to investigate the divalent cation requirement of PFV RT. The reverse transcription systems were set up as described previously in this letter, except that the bivalent cation was added to give a gradient with final concentrations of 1, 3, 5, 8, and 10 mmol/L Mg^{2+} and 0.1, 0.5, 1.0, 2.0, 3.0, and 3.5 mmol/L Mn^{2+} (Khan A S, et al., 1999). The results showed that the optimum concentration of Mg^{2+} was 3 mmol/L (Figure 1E) at which the maximum level of 5.54×10^6 pU/μL of PFV virions was detected (Figure 1F), while the optimum concentration of Mn^{2+} was 1 mmol/L (Figure 1G) with 5.12×10^7 pU/μL of PFV virions detected (Figure 1H). This confirmed that there was a preference of the PFV RT for Mn^{2+} rather than Mg^{2+}, which is similar to some oncogenic RNA viruses such as Rauscher leukemia virus and simian sarcoma virus (Liu W T, et al., 1977). Thus, whether PFV has the potential to induce tumors in its natural host deserves further research, and the results indicate that more attention should be paid to the connections between the different divalent cation requirements and the biological properties of the retroviruses.

After the temperature for PFV RT and the concentrations of Mg^{2+} or Mn^{2+} were optimized, a series of 10-fold dilutions of PFV lysates were prepared to verify the linearity of the SG-PERT assays. Favorable linearity and amplification efficiency were obtained in both Mg^{2+}-based and Mn^{2+}-based SG-PERT assays (Figure 1I, J) The PFV RT activity of the original stock, which was calculated according to the standard curve set up previously, was 1.55×10^8 pU/μL with 3 mmol/L Mg^{2+}, and 1.59×10^9 pU/μL with 1 mmol/L Mn^{2+}, indicating that the sensitivity of Mn^{2+}-based SG-PERT assay for PFV detection was about 10 times higher than that of Mg^{2+}.

In summary, we have established a modified SG-PERT assay for quantification of retroviral PFV. Unlike the Taqman PERT assay, which uses cDNA-specific probes with real-time PCR, the SG-PERT assay uses the more convenient and cost-efficient SYBR Green I and

has no inappropriately high fluorescence background as might be caused by probe degradation (Lovatt A, et al., 1999; Brorson K, et al., 2001). Although the separation of the reverse transcription and real-time PCR reactions employed in the SG-PERT assay was time-consuming, it helped to remove non-RT RNA templates and avoid background signals of the Taq polymerase compared with one-step SG-PERT.

The SG-PERT assay has some limitations. For example, it might not distinguish between infective and defective virions. In addition, the results of the PERT assay are not as intuitive, as the titer of PFV has to be converted from the activity of the RT. In further studies, other traditional quantification methods, such as indicator cell line, CPE observation, or western blotting, electron microscopy will be investigated to set up a comprehensive correlation with SG-PERT. Nevertheless, given its linearity over seven orders of magnitude, its low intra-assay and inter-assay CVs, high sensitivity, and relatively inexpensive reagents, the SG-PERT assay represents an ideal tool to determine the amount of PFV for both molecular biology research and clinical applications.

FOOTNOTES

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All of the authors declare that they have no competing interest. This article does not report on any studies with human or animal subjects.

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